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## Introduction

Selective depletion of intracellular oncogenic proteins is a potentially powerful tool for the treatment of breast cancer. This is usually achieved by genetic manipulation of the target gene using procedures such as gene disruption, antisense or ribozyme technologies. We now propose an alternative approach in which an oncogenic protein is specifically targeted for intracellular degradation. In order to do this we will take advantage of the permeability properties of the third helix of the antennapedia protein. This will be used to deliver a small trifunctional peptide consisting of a target protein binding peptide and a peptide designed to interact with the E2 class of ubiquitin conjugating enzymes. In this way the ubiquitin-conjugating machinery will be selectively recruited to the target protein, which should then be degraded by the proteasome. We will use the cytoplasmic signaling molecule  $\beta$ -catenin as one model system since its oncogenic activity is thought to be regulated at the level of protein stability and we have established that it is normally targeted for ubiquitination and proteosomal degradation. Mutations of  $\beta$ -catenin that increase its protein stability are oncogenic. The  $\beta$ -catenin binding peptide will be based on the region of the tumor suppressor protein APC that constitutively binds  $\beta$ -catenin. A second target will be ErbB-2 a tyrosine kinase strongly associated with breast cancer. The ErbB-2 binding peptide will consist either of the last three armadillo repeats of  $\beta$ -catenin known to constitutively bind ErbB-2 or the SH2 domain of grb2 which can only bind tyrosine phosphorylated ErbB-2. Generally, the work proposed is of great significance to the treatment of breast cancer. More specifically, proof of the principle that direct targeting of oncogenic proteins for intracellular degradation inactivates their transforming capabilities could lead to the development of novel therapeutic strategies based on this approach.

## Revised Statement of Work

The reviewer of year 2 of the Progress Report of my grant DAMD-98-1-8089 suggested that I provide a revised statement of work to more adequately incorporate the new data on I kappa B kinase. I have submitted this new SOW, which was recently approved. Since this grant was first submitted we have gathered important new information relating to the sites on  $\beta$ -catenin that regulate its phosphorylation, ubiquitination and degradation. In particular we found that IKK $\alpha$ , a kinase thought previously to only phosphorylate I $\kappa$ B proteins to regulate NF kappa B, is a potent and important kinase in the regulation of  $\beta$ -catenin phosphorylation and ubiquitination. In fact, our results point to IKK $\alpha$  rather than GSK-3 $\beta$  as the key enzyme in this process. This work was summarized in the original Progress Report. These results are extremely relevant to the present grant since they raise the possibility that sites other than, or in addition to, those we originally proposed to utilize in the construction of targeting vectors could be important. In addition, the results raise the possibility that vectors designed to target  $\beta$ -catenin may also interfere with NF-kappa B signaling. Clearly it is important that we understand the details of this important relationship. The revised Statement of Work now reflects this broadening of scope (see below). In addition we have applied for a non-funded extension of the grant so that we can complete the new experiments.

## Year 1

- a) Construction of at least 6 expression vectors containing erbB2 and  $\beta$ -catenin targeting, antennapedia and cyclin B destruction box fusion constructs. These will consist of vectors containing several different erbB-2 and  $\beta$ -catenin targeting sequences as well as cyclin B destruction boxes of various lengths and sequence. Our aim is to ascertain the minimum size of the final trifunctional peptide product that is effective in targeting erbB-2 and  $\beta$ -catenin for degradation.
- b) Co-express the vectors with erbB-2 or stable  $\beta$ -catenin (S37A mutant), investigate interaction with  $\beta$ -catenin.

## Year 2

- a) Investigate the role of the IKK complex in the regulation of  $\beta$ -catenin protein levels and signaling.
- b) Continue experiments with expression vectors. Monitor  $\beta$ -catenin ubiquitination and degradation.
- c) Adherex to make membrane permeant peptides and/or production of recombinant peptides.
- d) Test the ability of peptides to enter cell cytoplasm and/or nucleus.

## Years 3 and 4

- a) Identify IKK phosphorylation sites on  $\beta$ -catenin.
- b) Characterize the mechanism of IKK regulation of  $\beta$ -catenin signaling activity.
- c) Redesign the targeting peptides based on this information.
- d) Test the activity of the redesigned peptides on  $\beta$ -catenin for ubiquitination and degradation in vitro.
- e) Test the ability of peptides to inhibit growth and colony formation of  $\beta$ -catenin-transformed cells.

## Body:

In the first annual report we described in detail the progress we had made in constructing targeting vectors for ErbB-2. Briefly the key research accomplishments of the first 12 months were:

- 1) A number of different targeting vectors have been constructed.
- 2) The targeting constructs have been transfected into three different cells of varying ErbB-2 status
- 3) Two of the constructs yield protein products of the predicted size indicating that the recombinant peptides are stable and can be expressed at relatively high levels.
- 4) The constructs were detected with an antibody directed at the FLAG tag indicating that it is accessible and does not interfere with protein production.

In the second year of funding we concentrated on  $\beta$ -catenin. Since this grant was first submitted we gathered important new information relating to the sites on  $\beta$ -catenin that regulate its phosphorylation, ubiquitination and degradation. In particular we found that IKK $\alpha$ , a kinase thought previously to only phosphorylate I $\kappa$ B proteins, is a potent and important kinase in the regulation of  $\beta$ -catenin phosphorylation and ubiquitination. In fact, our results point to IKK $\alpha$  rather than GSK-3 $\beta$  as the key enzyme in this process (see report for year 2). These results are extremely relevant to the present grant since they raise the possibility that sites other than, or in addition to, those we originally proposed to utilize in the construction of targeting vectors could be important. This work was summarized in the previous report.

## Results for year 3

In the third year we continued our work on the Wnt/ $\beta$ -catenin IKK pathways. This work was carried out in collaboration with the laboratories of Richard Gaynor at UT Southwestern and Dr. Richard Pestell at Albert Einstein. We show a requirement for IKK $\alpha$  in PI3K-dependent induction of cyclin D1 and using cells from mice homozygously deleted of the *cyclin D1* gene, show a requirement for cyclin D1 in PI3K-dependent cellular proliferation. Since IKK $\alpha$  but not IKK $\beta$  induced cyclin D1 and  $\beta$ -catenin/Tcf activity, these studies indicate that relative abundance of IKK $\alpha$  and IKK $\beta$  alters their substrate and signaling pathway specificity. The divergent effects of IKK $\alpha$  and IKK $\beta$  on  $\beta$ -catenin/Tcf signaling suggest the IKK complex may play a key role in coordinating the Wnt pathway. The demonstration that IKK interacts directly with and phosphorylates  $\beta$ -catenin to regulate the

expression of growth promoting genes such as cyclin D1 is clearly very important in breast cancers that associated with cyclin D1 overexpression (ref).

### **Cyclin D1 is required for PI3K-dependent S-phase entry in primary cells.**

Activation of phosphatidylinositol (PI) 3'-kinase (PI3K) mediates signaling induced by a number of growth factors and tumor promoters and is required for mitogenic stimulation by specific growth factors during the G<sub>1</sub>-S phase of the cell-cycle<sup>24, 27</sup>. The role of PI3K in serum-induced cyclin D1 expression was examined in mouse embryonic fibroblasts (MEFs). In wildtype MEFs, cyclin D1 protein levels were elevated by 3 hrs after serum stimulation and the PI3K inhibitor, LY294002, abrogated the induction (Fig. 1a). Total ERK levels were unchanged and cyclin D1 was not induced by serum, however LY294002 reduced cyclin E levels by 30%. *Cyclin D1*<sup>-/-</sup> MEFs of identical passage (Fig. 1a) showed similar levels of total ERK and a delayed decrease in cyclin E levels (60%) 12 hrs after serum addition (not shown). Western blot analysis to detect the phosphorylation status of Akt showed an induction of Akt phosphorylation at 15 min and 3 hrs after serum stimulation and a 60% reduction with LY294002 at 2 hrs in both *CD1*<sup>-/-</sup> and *CD1*<sup>+/+</sup> MEFs (not shown). In wildtype MEFs, serum induced entry into the DNA synthetic phase with the S-phase fraction increasing from 10% to 54% (Fig. 1b). LY294002 reduced the S-phase proportion from 23% to 7% at 24 hrs, and from 54% to 5% at 48 h, indicating that serum-induced DNA synthesis is PI3K-dependent in MEFs. Serum-induced DNA synthesis was reduced to 14% in the *cyclin D1*<sup>-/-</sup> MEFs (Fig. 1b). Thus cyclin D1 is required for PI3K-dependent induction of DNA-synthesis by serum. To determine the role of PI3K in apoptosis mediated by serum deprivation, annexin V staining and sub G<sub>1</sub> analysis was performed on the MEFs. In the basal state, *cyclin D1*<sup>-/-</sup> MEFs exhibited a 5-fold greater level of annexin V staining, indicating increased apoptosis which was rescued by serum (Fig. 1c). LY294002 did not affect the level of cellular apoptosis in either wt or *cyclin D1*<sup>-/-</sup> MEFs as determined by either annexin V staining (Fig. 1c) or sub G<sub>1</sub> analysis (not shown). Similar analyses were performed on serum-induced DNA synthesis in 3T3 cells derived from the *CD1*<sup>+/+</sup> and *cyclin D1*<sup>-/-</sup> MEFs. Serum-induced S-phase entry was inhibited by LY294002 in *CD1*<sup>+/+</sup>, whereas S-phase was poorly induced in the *cyclin D1*<sup>-/-</sup> 3T3 cells and LY294002 had no effect (Fig. 1d). Together these findings suggest serum-induced DNA synthesis requires PI3K and that a substantial proportion of PI3K-dependent induction of DNA synthesis requires cyclin D1. Other mechanisms of S-phase entry must exist in the *cyclin D1*<sup>-/-</sup> cells, which are likely dependent on cyclin E. PI3K signaling does not play a dominant role in the increased apoptosis induced by serum deprivation in *cyclin D1*<sup>-/-</sup> MEFs.

### **PI3K-induction of cyclin D1 is dependent upon IKK $\alpha$ .**

Type 1 PI3K is a heterodimeric holoenzyme, consisting of regulatory (p85) and catalytic (p110) subunits that was initially identified through its role in Src-mediated transformation. Since the induction of cyclin D1 protein levels by serum was PI3K sensitive, and because activation of PI3K and Akt plays a key role in DNA synthesis in prostate cancer cells<sup>25, 26</sup>, we examined whether the cyclin D1 promoter was directly induced by constitutively active PI3K (p110 $\alpha$ -CAAX). Oncogenic forms of p110 $\alpha$  and p85 have been identified and expression of constitutively active PI3K triggers DNA synthesis<sup>24, 29</sup>. We examined the role of PI3K in the PTEN-containing prostate cancer cell line DU145. Cyclin D1 abundance was induced 4-fold by serum addition and the induction was reduced 45% by LY294002 (data not shown). The cyclin D1 promoter (-1745 CD1LUC) was induced 2.5-fold by p110 $\alpha$ -CAAX while the kinase dead (p110 $\alpha$ -CAAX KD) mutant was inactive (Fig. 2a). The constitutively active p110 $\alpha$ -K227E mutant induced cyclin D1 2.2-fold (Fig. 2a). Cyclin D1 protein levels in cells transfected with the p110 $\alpha$ -CAAX vector were induced 3-fold (not shown). The PI3K responsive *c-fos* promoter was induced 10-fold (data not shown), but the cyclin E and cyclin A promoters were not (Fig. 2b), suggesting the induction of cyclin D1 is not an indirect effect of PI3K activity on DNA-synthesis and is promoter specific. Since cryptic activation sequences, including AP-1, have been identified in several expression vectors, we examined the empty luciferase reporter pA3LUC in which the cyclin D1 promoter was cloned and found that pA3LUC was not induced with



pGL3LUC was induced 3-fold by p110 $\alpha$ -CAAX (Fig. 2b). Cyclin D1 promoter activation by p110 $\alpha$ -CAAX inhibited by either LY294002 (Fig. 2c) or Wortmannin (not shown), by dominant negative mutants of the PI regulatory subunit (Fig. 2d), an N17Rac mutant and a kinase inactive dominant negative Akt (Akt K179M) but by wt Akt (Fig. 3d). The finding that Rac1-N17 blocked PI3K-induced activity is consistent with previous studies<sup>30</sup>. Since activating mutations of Rac1 induce both NF- $\kappa$ B activity and the *cyclin D1* gene through an NF- $\kappa$ B dependent pathway<sup>31</sup>, we examined the possibility that NF- $\kappa$ B activity may play a role in PI3K induction of cyclin D1 using dominant I $\kappa$ B inhibitor [CMV-I $\kappa$ B]. CMV-I $\kappa$ B $\alpha$ Sr inhibited p110 $\alpha$ -CAAX-induced activation of cyclin D1 (Fig. 2d) but did not inhibit *c-fos* LUC activity (not shown). The p38 MAPK inhibitor SB203580, the ERK inhibitor PD98059 and rapamycin did not effect p110 $\alpha$ -induced D1 activity (not shown). Both the dominant negative and kinase dead IKK $\alpha$  reduced PI3K-induced cyclin D1 promoter activity and basal promoter activity in a dose-dependent and promoter specific manner (Fig. 2e). Since the inhibition by I $\kappa$ B $\alpha$ Sr suggested that p110 $\alpha$ -CAAX induction of cyclin D1 may involve IKK/NF- $\kappa$ B activity, we assessed the effect of p110 $\alpha$ -CAAX on the  $\kappa$ B-responsive reporter 3 $\kappa$ BLUC. p110 $\alpha$ -CAAX induced 3 $\kappa$ BLUC activity 3-fold which was inhibited by either LY294002 (Fig. 2f) or the dominant inhibitor CMV-I $\kappa$ B Sr (not shown).

### **IKK $\alpha$ , but not IKK $\beta$ induces cyclin D1 through $\beta$ -catenin/Tcf.**

The studies described above indicate that the PI3K activation of cyclin D1 involves Akt and I $\kappa$ B, consistent with studies demonstrating that PI3K/Akt can activate NF- $\kappa$ B signaling<sup>32</sup>. The constitutively active mutant IKK $\alpha$ CA(S176/180E), induced the cyclin D1 promoter 4-fold (Fig. 3a), while the constitutively active mutant IKK $\beta$  (IKK $\beta$ CA) decreased promoter activity (see below). To determine the IKK $\alpha$  responsive element in the cyclin D1 promoter, we first examined elements contributing to basal enhancer activity in the cyclin D1 promoter, which contains several known transcription factor binding sites (eg. CRE, Tcf and NF- $\kappa$ B), that convey cell-type basal activity<sup>33</sup>. Point mutation of the CRE site or the Tcf site reduced basal cyclin D1 promoter activity by 20% and 55%, respectively (Fig. 3b). In contrast, mutation of the NF- $\kappa$ B binding site enhanced basal level activity (data not shown), suggesting that the NF- $\kappa$ B is a negative regulator of cyclin D1 in DU145 cells. Since the CRE and Tcf sites together contribute to the majority of the cyclin D1 promoter activity in DU145 cells, we assessed the role of these elements in the activation of the cyclin D1 promoter by IKK $\alpha$ . Mutation of either of these sites abolished induction of cyclin D1 by IKK $\alpha$ CA (Fig. 3c) (mutation of the NF- $\kappa$ B site had no effect on activation by IKK $\alpha$ CA (not shown)). IKK $\alpha$ CA also induced TOP-LUC (a reporter construct that contains multimerized sequences identical to the cyclin D1 Tcf site and which reflects  $\beta$ -catenin activity), 3-fold, similar to the activation of the canonical NF- $\kappa$ B responsive sequences (3 $\times$ RelLUC) (Fig. 3c). The divergent effects of IKK $\alpha$  and IKK $\beta$  (see below Fig. 4) on cyclin D1 raises the possibility that these kinases may be engaged by distinct upstream activators. We investigated whether NIK served as an upstream MAPKKK. NIK induced 3 $\times$ RelLUC 20-fold, as previously shown<sup>34</sup> while the cyclin D1 was repressed by NIK (Fig. 3d). Conversely, a dominant negative NIK (KK429/430AA) inhibited 3 $\times$ RelLUC and activated cyclin D1 (Fig. 3d), suggesting that the MAPKKK(s) that regulate NF- $\kappa$ B and cyclin D1 activity are distinct. Next we compared the activity of NIK in SW480 cells, which contain a truncated APC gene and, as a result, have increased  $\beta$ -catenin levels and signaling. In SW480 cells, wt NIK induced 3 $\times$ RelLUC activity, repressed Tcf reporter activity (Fig. 3e). Since NIK plays a critical role in TNF $\alpha$ -dependent induction of NF- $\kappa$ B activity<sup>32</sup>, we examined the effect of TNF $\alpha$  on the 3 $\times$ Rel reporter plasmid and found that TNF $\alpha$  induced 3 $\times$ RelLUC but repressed Tcf activity (Fig. 3f). It appears, therefore, that different IKK kinases regulate IKK activity, resulting in distinct downstream signaling events. Overexpression of wt APC inhibited Tcf reporter activity and overexpression of a constitutively active  $\beta$ -catenin ( $\beta$ -catenin S37A) rescued the repression by APC (Fig. 3h), consistent with previous studies of Tcf reporter activity and induction of cyclin D1 by  $\beta$ -catenin in these cells<sup>8</sup>. Thus IKK $\alpha$  activates Tcf signaling and the *cyclin D1* gene through a mechanism that is distinct from the pathway by which TNF $\alpha$  and NIK regulate NF- $\kappa$ B activity.

Next we examined the ability of IKK $\beta$  to regulate Tcf activity. Constitutively active IKK $\beta$  (IKK $\beta$ CA) increased NF- $\kappa$ B reporter activity, but in contrast to IKK $\alpha$ ,  $\beta$ -catenin signaling was decreased by 80-90% (Fig. 4). Furthermore, a kinase dead IKK $\beta$  mutant did not affect  $\beta$ -catenin activity (Fig. 4a), indicating that intact kinase function is necessary for IKK $\beta$  to affect  $\beta$ -catenin signaling. IKK $\beta$ CA also decreased the activity of a known  $\beta$ -catenin target gene, the cyclin D1 promoter construct, by 90% in SW480 cells (-163CD1Luc contains the function Tcf/LEF sites as well as CREB, AP-1, Sp1 and NF- $\kappa$ B sites) (Fig. 4b). In contrast, a cyclin D1 promoter with mutated Tcf/LEF site but which retained the other regulatory elements, was not responsive to IKK $\beta$  (Fig. 4b). The Tcf sequence was also sufficient for repression by IKK $\beta$ CA when coexpressed in keratinocytes (Fig. 4c). Together these results indicate that IKK $\alpha$  activation has a direct effect on the activity of the IKK complex toward  $\beta$ -catenin signaling.

### **IKK $\alpha$ associates with and phosphorylates $\beta$ -catenin and increases $\beta$ -catenin abundance.**

Several lines of evidence suggest that IKK $\alpha$  and IKK $\beta$  fulfill distinct functions. For example, homozygous deletion of the *IKK $\alpha$*  and *IKK $\beta$*  genes results in distinct phenotypes<sup>15, 17, 18, 35</sup>. Furthermore, IKK $\beta$  has high activity for the I $\kappa$ B proteins and has a more significant role in the NF- $\kappa$ B pathway in response to activation with TNF $\alpha$  and IL-1 than does IKK $\alpha$ <sup>15, 16, 36</sup>. To investigate the possible mechanisms responsible for these diverse functions, we assessed the subcellular localization of IKK $\alpha$  and IKK $\beta$  as well as cell type expression pattern. Western blot analysis of nuclear and cytoplasmic extracts confirmed the differential localization of IKK $\alpha$  and IKK $\beta$  in Cos-7 cells (Fig. 5a). SKBR3 and SW480 cells which are of epithelial derivation, expressed significantly more IKK $\alpha$  than IKK $\beta$ , whereas Jurkat lymphocytic cells, expressed similar levels of IKK $\alpha$  and  $\beta$  (not shown). We hypothesize that this differential localization of the two IKK's is relevant for the precise regulation of both nuclear and cytoplasmic functions of I $\kappa$ B and  $\beta$ -catenin. Since IKK $\alpha$ CA induced cyclin D1 and Tcf reporter activity in SW480 cells, we hypothesized that IKK $\alpha$  may regulate  $\beta$ -catenin abundance and/or phosphorylation. To determine whether IKK $\alpha$  could directly interact with  $\beta$ -catenin, immunoprecipitation was performed in Cos-7 cells co-transfected with HA-tagged  $\beta$ -catenin and FLAG-tagged IKK $\alpha$ .  $\beta$ -catenin is present in IKK $\alpha$  immunoprecipitates and IKK $\alpha$  is also present in  $\beta$ -catenin immunoprecipitates (Fig. 5b). Endogenous association between  $\beta$ -catenin and IKK $\alpha$  was demonstrated by reciprocal IP-western blotting in SW480 cells (Fig. 5c). The total  $\beta$ -catenin level, as well as the amount of a higher molecular weight form of  $\beta$ -catenin and the level of the  $\beta$ -catenin S37A mutant, were all increased in cells co-expressing IKK $\alpha$ CA and  $\beta$ -catenin expression vectors (Fig. 5d) consistent with a role for IKK $\alpha$  in regulating  $\beta$ -catenin phosphorylation and/or abundance. Point mutation of  $\beta$ -catenin at S33 to alanine abrogated the induction of the higher molecular weight form of  $\beta$ -catenin. In the presence of IKK $\alpha$ CA,  $\beta$ -catenin abundance is increased and migrated as two distinct bands, with the slower migrating species likely representing either a phosphorylated or mono-ubiquitinated form<sup>37</sup>. A phospho- $\beta$ -catenin antibody demonstrated an induction of phosphorylated  $\beta$ -catenin forms in the presence of IKK $\alpha$ CA and the loss of the phosphorylated higher molecular weight form of S33A- $\beta$ -catenin (Fig. 5e). Since GSK3 $\beta$  phosphorylation of  $\beta$ -catenin results in a reduction of  $\beta$ -catenin levels, the increase in  $\beta$ -catenin levels by IKK $\alpha$ CA suggests that IKK regulation of  $\beta$ -catenin is distinct from GSK3 $\beta$ . Consistent with findings in cultured cells, we found that  $\beta$ -catenin fusion proteins were efficient substrates for phosphorylation by IKK $\alpha$  *in vitro* and the minimal region involved the N-terminal portion between aa 30 and 55 (Fig. 5f). IKK $\alpha$  bound to and phosphorylated  $\beta$ -catenin *in vitro* with efficiency that is similar to that of I $\kappa$ B (not shown). The findings that IKK $\alpha$  phosphorylates  $\beta$ -catenin and that IKK $\alpha$ CA increases Tcf activity and  $\beta$ -catenin abundance, suggests that  $\beta$ -catenin phosphorylation by IKK contributes to a novel pathway of  $\beta$ -catenin-mediated Tcf-dependent gene transcription (Fig. 5g).



## Figure Legends

**FIG. 1. The PI3-kinase induction regulates cyclin D1 and S-phase entry.** (A). Western blot analysis of mouse embryo fibroblasts (MEFs) from cyclin D1 wild type (CD1wt) or CD1<sup>-/-</sup> mice and (B) DU145 cells, were treated with serum either with or without the PI3K inhibitor LY294002. Western blot was performed for cyclin D1, Akt, total ERK and cyclin E. (C). FACS and (D) apoptosis analyses were performed of serum released CD1wt and CD1<sup>-/-</sup> MEFs or (E) NIH3T3 cells treated either with vehicle or LY294002 (X $\mu$ M).

**FIG. 2. The cyclin D1 promoter is induced by PI3K** (A). DU145 cells were transfected with the cyclin D1 promoter luciferase reporter plasmid (-1745 CD1LUC) and the p110 $\alpha$ -CAAX expression plasmid in 10% or 0.5% serum. The -fold induction of luciferase reporter activity is shown for 9 separate experiments as mean  $\pm$  S.D. throughout. (B). The effect of the p110 $\alpha$  kinase dead mutant (p110 $\alpha$ -CAAX-KD) or (C) constitutively active p110 $\alpha$ -K227E mutant on -1745 CD1LUC was assessed. (D) The effect of the p110 $\alpha$ -CAAX expression plasmid was assessed in conjunction with reporter plasmids for cyclin A, cyclin E, and the luciferase reporter pA<sub>3</sub>LUC and pGL3LUC.

**FIG. 3. PI3K-induction of cyclin D1 involves IKK $\alpha$ , I $\kappa$ B.** (A). The p110 $\alpha$ -CAAX induced cyclin D1 promoter activity was inhibited by LY294002 (from x $\mu$ M to x mM) in either the presence or (B) absence of p110 $\alpha$ -CAAX. (C). p110 $\alpha$ -CAAX induced cyclin D1 promoter activity was reduced by dominant negative inhibitors of PI3K (schematic) or by (D). coexpression of RacN17 or AktDN (K179M) but was not reduced by addition of chemical inhibitors of the MAPK pathways. The I $\kappa$ B $\alpha$ SR and PDTC (x $\mu$ M) are inhibitors of NF $\kappa$ B signaling. (E). The NF $\kappa$ B-luciferase reporter gene was induced by p110 $\alpha$ -CAAX and inhibited by (F). LY294002 in a dose-dependent manner.

**FIG. 4. The IKK $\alpha$ , but not IKK $\beta$  pathway induces cyclin D1.** (A). The p110 $\alpha$ -CAAX induced activity of the -1745 CD1LUC reporter was reduced by co-expression of the IKK $\alpha$  KM (K54M) or IKK $\alpha$ DN (S176/A) mutant. (B). The IKK $\alpha$  inhibitory mutants reduced cyclin D1 basal activity 50- to 60%. (C). The IKK $\alpha$ CA(S176E) mutant activated the cyclin D1 promoter (D), requiring the CRE and TCF sites. (E). Schematic representation of the cyclin D1 promoter, with the sequences homologous to CRE and Tcf binding sites indicated. (F). The IKK $\alpha$ CA(S176E) induced the heterologous reporter encoding the Tcf site and the NF $\kappa$ B reporter. (G). The IKK activating kinase NIK, induced NF $\kappa$ B reporter activity, but repressed the cyclin D1 promoter. The NIKDN(AA) inhibited the NF $\kappa$ B-reporter and induced the cyclin D1 promoter, indicating the IKK $\alpha$  kinase inducing cyclin D1 is not NIK. (H) SW4 cells were co-transfected with 0.1  $\mu$ g APC and 0.1  $\mu$ g  $\beta$ -catenin S37A in addition to TOP-LUC and Renilla. APC decreases  $\beta$ -catenin signaling by 80%.  $\beta$ -catenin S37A inhibited the ability of APC to decrease  $\beta$ -catenin signaling. (I) 3xRel-LUC or FOPLUC reporters were coexpressed in SW480 cells with either NIKwt or (J) treated with TNF $\alpha$ . The data are mean  $\pm$  SEM.

**FIG. 5. IKK $\beta$  induces NF $\kappa$ B but inhibits TCF activity.** (A). Substantially more IKK $\alpha$  than IKK $\beta$  was found in the nuclear extracts (NE) of Cos-7 cells. Internal controls for (nuclear) TFIIB and cytoplasmic markers are shown. (B). The constitutively active IKK $\beta$ CA(SE) inhibited activity of the TOP-LUC reporter and induced activity of the NF $\kappa$ B reporter (3xRel-LUC). (C). The cyclin D1 promoter was repressed by IKK $\beta$ CA(SE) and deletion of the site abrogated repression. (D). The kinase inactive mutation of IKK $\alpha$  or IKK $\beta$  abrogated *trans*regulation of the site. (E). The IKK $\beta$ CA(SE) inhibited Tcf-LUC activity in SKBR3 and (F) human epidermal keratinocytes inhibited NF $\kappa$ B-reporter activity.

**Fig. 6. IKK $\alpha$  associates with and phosphorylates  $\beta$ -catenin and increases  $\beta$ -catenin abundance.** (A). SW4 cells were transfected with the constitutively active IKK $\alpha$  (IKK $\alpha$ CA(S/E)) and -1745 CD1LUC reporter or T

LUC. Fold induction is shown compared with equal amounts of empty expression vector cassette. (B). The effect of the proteasome inhibitor ALLN on the IKK $\alpha$ CA(S/E) induced activity of TOP-LUC shows a 2-to 3-fold increase. (C) Immunoprecipitation studies show that  $\beta$ -catenin associates with IKK $\alpha$  when either protein is precipitated from cells transfected with Flag-IKK $\alpha$  and HA- $\beta$ -catenin. (D). In cultured SW480 cells endogenous associations are found between IKK $\alpha$  and  $\beta$ -catenin by immunoprecipitation western blot analysis. (E) The IKK $\alpha$ CA(S/E) expression plasmid was coexpressed in cells transfected with either wild type or mutants of (S33A, S37A) of  $\beta$ -catenin. Western blotting shows an increase in the total amount of  $\beta$ -catenin, including a higher molecular weight form (2 arrows). The S33A $\beta$ -catenin shows no increase in the amount of the high molecular weight form. (F) Western blot with phosphospecific  $\beta$ -catenin antibodies confirms the presence of phosphorylated  $\beta$ -catenin including the higher molecular weight form, and the failure of this complex to form with the S33A  $\beta$ -catenin mutant. (G). GST- $\beta$ -catenin fusion proteins were used in IP-kinase assay with IKK $\alpha$ . The phosphorylated forms of  $\beta$ -catenin are shown, together with the coomassie stained gel of the fusion proteins. (H). Model by which IKK $\alpha$  phosphorylates  $\beta$ -catenin at S33 to induce Tcf signaling of target genes including cyclin D1.

Figure 1

24hrs-LY

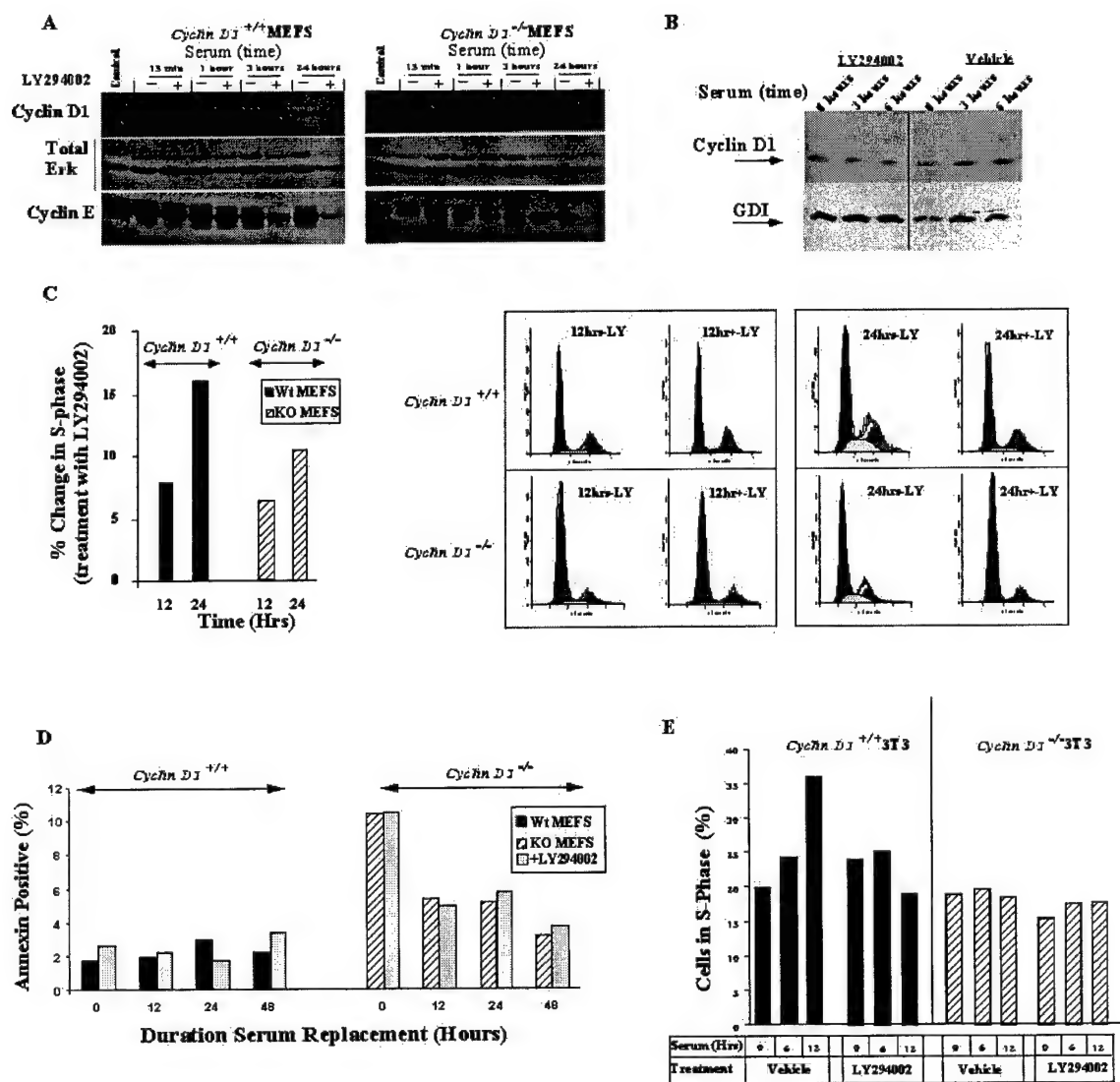


Figure 2

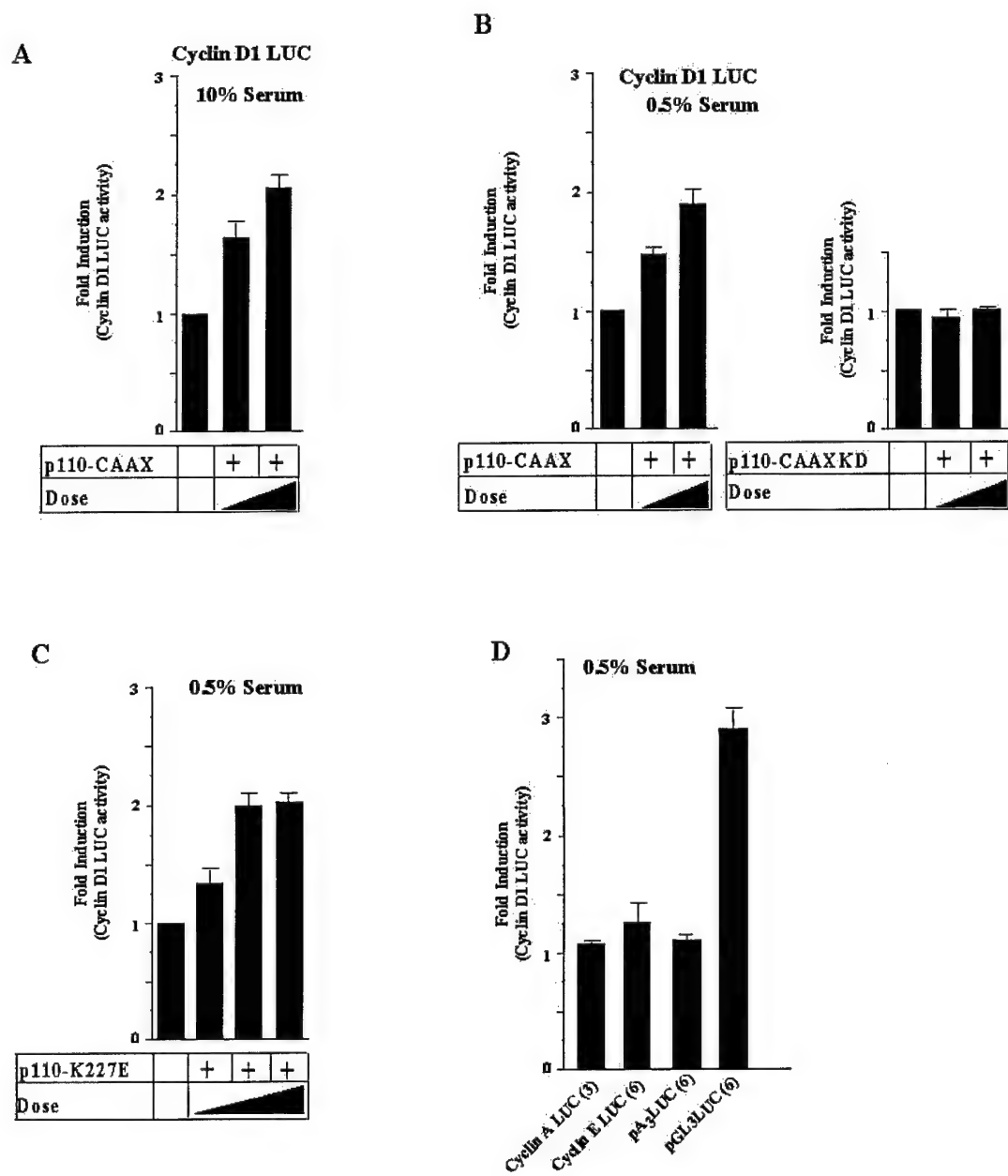


Figure 3

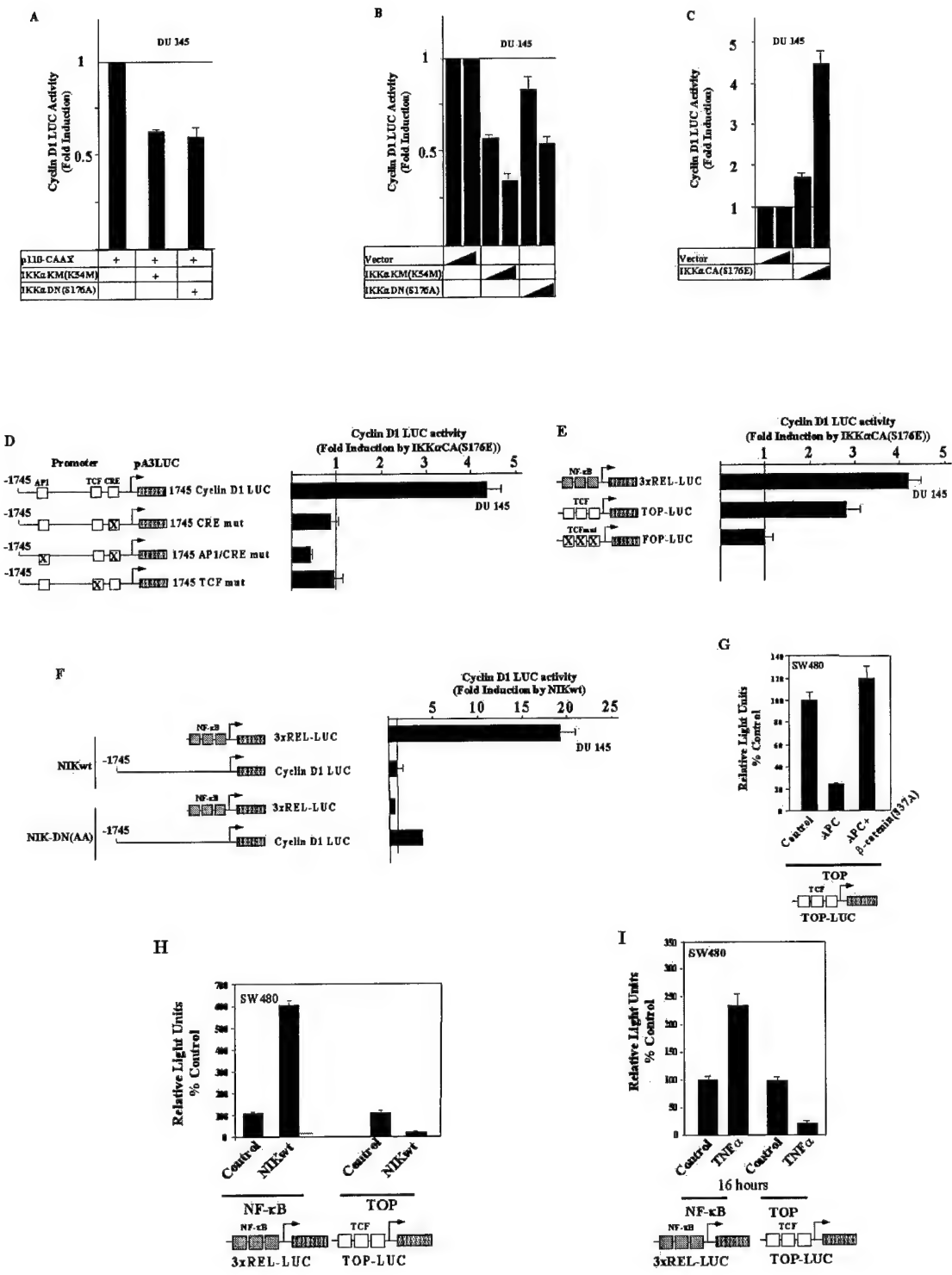




Figure 4

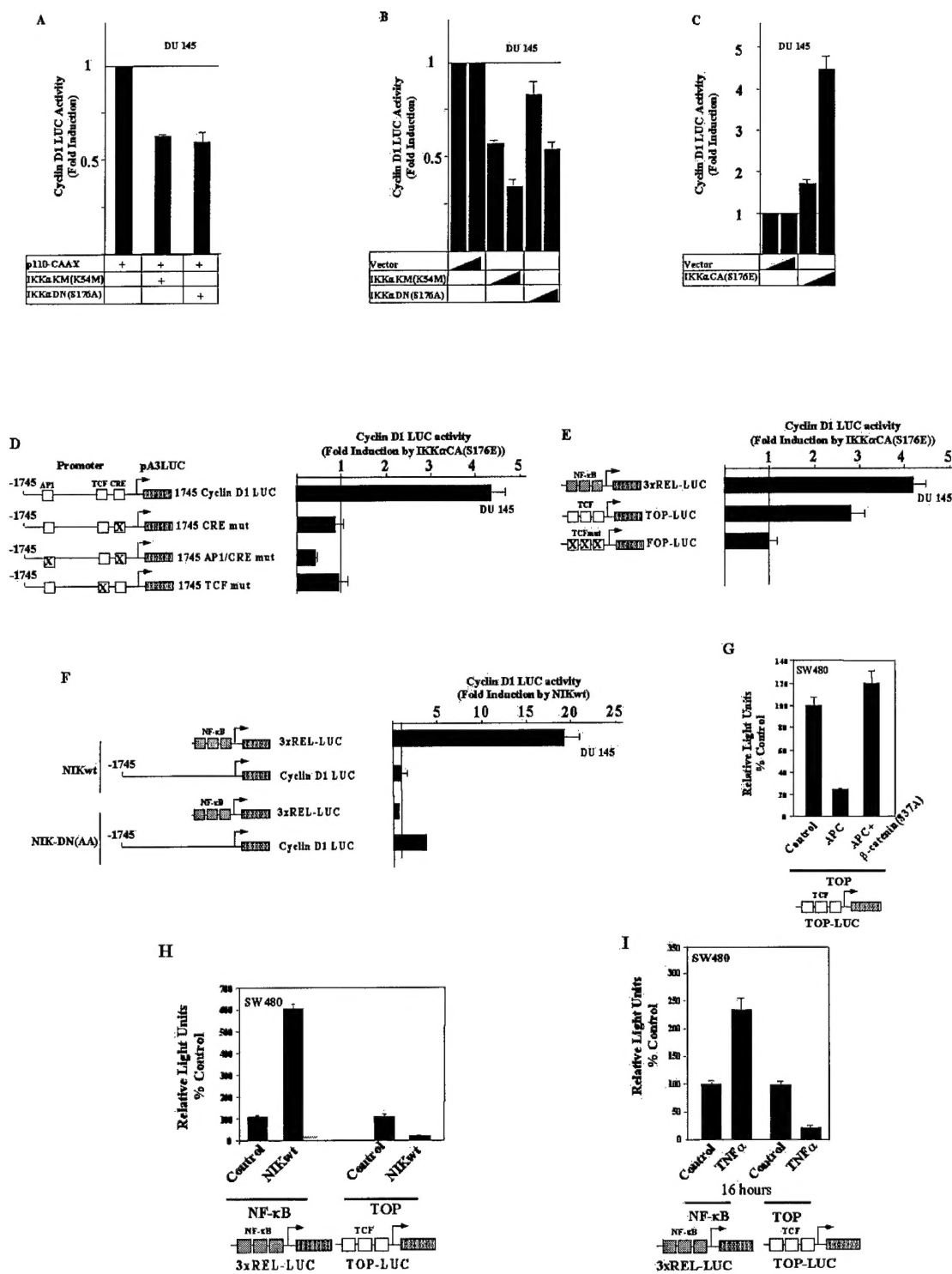


Figure 5

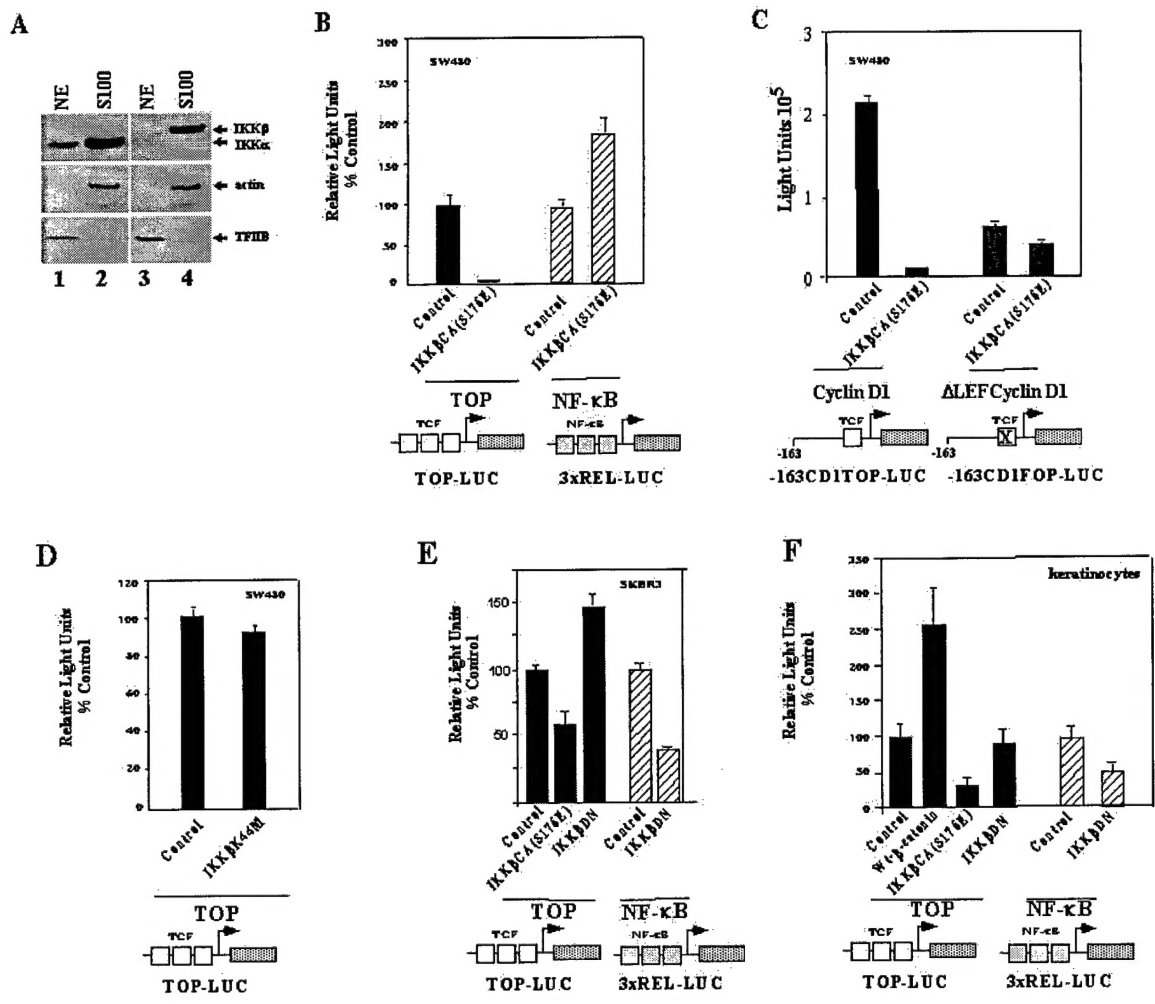
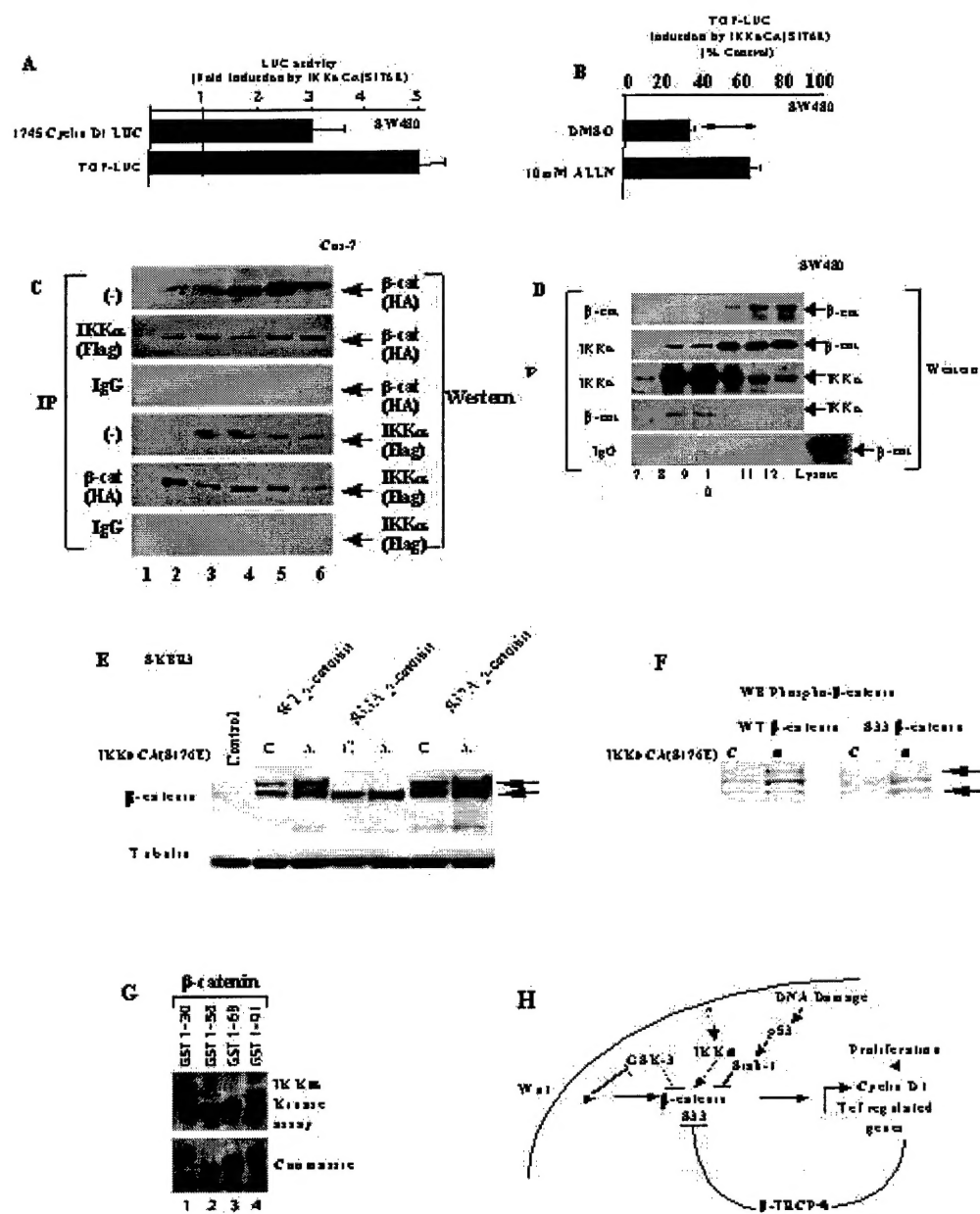


Figure 6



## Key Research Accomplishments:

1. Cyclin D1 is required for PI3K-dependent S-phase entry in primary cells.
2. PI3K-induction of cyclin D1 is dependent upon IKK $\alpha$ .
3. IKK $\alpha$ , but not IKK $\beta$  induces cyclin D1 through  $\beta$ -catenin/Tcf
4. IKK $\alpha$  associates with and phosphorylates  $\beta$ -catenin and increases  $\beta$ -catenin abundance.

## Reportable outcomes:

IKK $\alpha$  activates Tcf signaling through an interaction with and phosphorylation of  $\beta$ -catenin. C Albanese\*, CR. Jarrett<sup>†</sup>, F Attiga<sup>†</sup>, D Joyce\*, J Hughes\*, J Hult\*, T Sakamaki\*, K Wu\*, M D'Amico\*, M Fu\*, A Ben-Ze'ev<sup>‡</sup>, C Lamberti<sup>¶</sup>, K-M Lin<sup>¶</sup>, RB. Gaynor<sup>¶</sup>, SW. Byers<sup>†</sup> and RG. Pestell. Submitted

## Conclusions:

This study demonstrated a novel pathway involving IKK $\alpha$  in the  $\beta$ -catenin-mediated regulation of activity. Endogenous IKK $\alpha$  was found in association with  $\beta$ -catenin in cultured cells while a constitutively active form of IKK $\alpha$  increased both  $\beta$ -catenin abundance and phosphorylation and induced Tcf-dependent transcription. This effect of IKK $\alpha$  on  $\beta$ -catenin was dependent, at least in part, on the S33 residue of  $\beta$ -catenin. Tight regulation of the  $\beta$ -catenin/Tcf pathway is crucial for normal development. Aberrant activation of the Tcf pathway contributes to the development of a variety of human cancers, including colon, breast and prostate cancer. Mutations in components of the Wnt-signaling pathway are commonly observed in human cancer and result in the accumulation of  $\beta$ -catenin and activation of Tcf/Lef target genes. Wnt family ligands and frizzled family receptors define one important mechanism that can induce  $\beta$ -catenin/Tcf signaling. In addition, suppressor screens in *Drosophila* have identified *Dpresenilin* as a target of *Armadillo* (a homolog of  $\beta$ -catenin) and cell-adhesion dependent pathway involving the integrin linked kinase (ILK) can also control  $\beta$ -catenin levels and activity. The *cyclin D1* gene which plays a critical role in oncogenic signaling pathways, is regulated (via its Tcf site) by several of these component pathways which regulate  $\beta$ -catenin/Tcf signaling. Thus, in addition to this study, in which the *cyclin D1* gene was induced by IKK $\alpha$ , previous studies have demonstrated the activation of the *cyclin D1* gene by stable mutants of  $\beta$ -catenin, Wnt-1, ILK and repression by presenilin 1. Cyclin D1 and  $\beta$ -catenin over-expression correlates with poor prognosis in human breast cancer suggesting a role for cyclin D1 in  $\beta$ -catenin/Tcf signaling and transformation. Understanding the relative impact of each of the pathways regulating  $\beta$ -catenin/Tcf activity and the effectors of cellular growth is fundamental to understanding dysregulation of this pathway in cancer.